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Human Papillomavirus E7 Oncoproteins Bind a Single Form of Cyclin E

MARITZA C. MCINTYRE,*,[1] MARGARET N. RUESCH,* and LAIMONIS A. LAIMINS*,†,[2]

*Departments of *Microbiology–Immunology and †Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Chicago, Illinois 60611*

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The E6 and E7 proteins of the high-risk human papillomaviruses (HPVs) act coordinately to immortalize human keratinocytes. These viral oncoproteins function by binding and altering the activity of cellular proteins which regulate cell cycle progression. Among the proteins bound by E7 are the retinoblastoma protein, Rb, as well as the related p107 and p130 proteins. In addition, E7 binds cyclin A, which regulates transit through the S and G2/M phases of the cell cycle. In this study, we demonstrate that HPV 18 E7 also associates with cyclin E which controls the G1/S transition. E7/cyclin E complexes were immunoprecipitated from E7-expressing cells as well as from cell extracts using GST–E7 fusion proteins. E7 was found to complex with a single form of cyclin E, and the binding was mediated through p107. Both E7/cyclin E and E7/cyclin A complexes exhibit kinase activity through associated cdk2 proteins which can contribute to phosphorylation of p107. The association of E7 with proteins which regulate transit through the cell cycle may provide an additional mechanism by which infection with human papillomaviruses results in cellular hyperproliferation.

INTRODUCTION

Human papillomaviruses are the etiologic agents of cervical cancer (zur Hausen, 1985), and their major transforming functions are encoded by the E6 and E7 open reading frames. Both the E6 and E7 proteins are capable of transforming rodent fibroblasts (Bedell *et al.,* 1989; Edmonds and Vousden, 1989; Phelps *et al.,* 1988), and the coordinate action of both proteins is required for the immortalization of human keratinocytes (Kaur and McDougal, 1988; Munger *et al.,* 1989). The E6 protein binds the p53 protein and targets it for degradation through a ubiquitin-mediated pathway (Crook *et al.,* 1991; Scheffner *et al.,* 1990). The E7 protein alters normal growth control by binding a set of cellular proteins including the retinoblastoma protein, Rb (Dyson *et al.,* 1989). Similar activities have been demonstrated for the adenovirus E1A protein and SV40 large T antigen (DeCaprio *et al.,* 1988; Whyte *et al.,* 1988) which share two regions of homology with E7, termed CR1 and CR2. These regions are essential for Rb binding as well as transformation. In nontransformed cells, E2F transcription factors associate with Rb in early G1 and dissociate upon phosphorylation of Rb in late G1 (reviewed in Nevins, 1992). This dissociation results in the activation of E2F-dependent genes which include those involved in DNA replication. The binding of viral oncoproteins to Rb results in the constitutive dissociation of E2F leading to the deregulated expression of E2F-responsive genes (Nevins, 1992). Mutants of E7 have been identified which are deficient for Rb binding but retain the ability to immortalize primary human keratinocytes (Jewers *et al.,* 1992). Additional studies have shown that E7 mutants which are unable to bind zinc but retain wild-type Rb binding activity are incapable of transforming cells in culture (McIntyre *et al.,* 1993). Taken together, these studies suggest that E7 may target other cell cycle-dependent processes.

The additional activities of E7 most likely involve interaction with other cellular proteins, such as p107 and p130 which are also involved in growth regulation (Dyson *et al.,* 1992; Ewen *et al.,* 1992; Li *et al.,* 1993). Both p107 and p130 bind members of the E2F family in a manner similar to Rb and contribute to the cell-cycle-dependent regulation of transcription (Schwarz *et al.,* 1993; Cobrinik *et al.,* 1993). Unlike Rb, p130 and p107 also bind and are phosphorylated by cyclin A–cdk2 and cyclin E–cdk2 complexes (Ewen *et al.,* 1992; Faha *et al.,* 1992; Hannon *et al.,* 1993; Lees *et al.,* 1992; Li *et al.,* 1993). Cyclin proteins activate a family of cyclin-dependent kinases at specific points in the cell cycle. While cyclin A activates cdk2 and cdc2 during S and G2/M phases, respectively, cyclin E activates cdk2 primarily during the G1/S transition (Hunter, 1993; Scherr, 1993). These cyclin complexes modulate the activity of other cell cycle regulators, such as Rb, through phosphorylation. Transforming proteins from the DNA tumor viruses specifically target cyclin complexes. While E1A associates with both cyclins A and E, T-antigen and E7 have only been reported to bind

[1] Current address: INSERM U370, 156 Rue du Vaugirard, Paris, France 75015.

[2] To whom correspondence and reprint requests should be addressed. Fax: (312) 503-1339.

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cyclin A (Adamczewski *et al.,* 1993; Arroyo *et al.,* 1993; Faha *et al.,* 1993; Pines and Hunter, 1990; Tommassino *et al.,* 1993). In this study, we have investigated whether the E7 protein from the high-risk HPV type 18 forms a complex with cyclin E which regulates transit from G1 to S.

MATERIALS AND METHODS

Mutagenesis of the CR1 and CR2 domains of HPV-18 E7. Mutations were introduced into the HPV-18 E7 gene by recombinant PCR techniques as described (McIntyre *et al.,* 1993) and the mutant sequences were cloned into the vector pSG5 to create CR1 mutant pS2H2P (amino acid 2 changed to proline) and the CR2 mutants pS27C2G (amino acid 27 changed to a glycine), pSΔDLLC (amino acids 24–27 are deleted), pSΔDLLC31F (amino acids 24–27 are deleted and 31 changed to phenylalanine), and pSΔDLLC20K (amino acids 24–27 are deleted and 20 changed to glutamic acid).

Construction of wild-type and mutant GST–E7 fusion expression constructs. The E7 gene was fused to the glutathione *S*-transferase gene to facilitate the one-step purification of the fusion protein (Smith *et al.,* 1988). pGSTE7 was constructed by digesting pETE7 (McIntyre *et al.,* 1993) with *Nco*I and *Eco*RV and ligating this fragment into the plasmid pGex-KG (Guan and Dixon, 1991), which had been digested with *Hin*dIII, filled in with Klenow, and then digested with *Nco*I. Plasmids pGST27C2G, pGSTΔDLLC, pGSTΔDLLC20K, and pGSTΔDLLC31F were constructed by amplifying the mutant E7-containing sequences from the constructs described above using synthetic oligonucleotides which add an *Nco*I site to the 5′ end of the gene and a *Bam*HI site to the 3′ end. The *Bam*HI site was filled in with Klenow and the fragment was cloned into pGex-Kg as described above.

Other expression plasmids. The GST–p107 fusion protein was expressed from the plasmid GST107 (23), a gift of Mark Ewen (Dana-Farber Cancer Institute, Boston, MA). The plasmid Gex-E (Koff *et al.,* 1991), which expressed a GST–cyclin E fusion, was a gift of James Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). The cyclin E gene was cloned into the plasmid pBluescript by digesting the cyclin E gene from GEX-E with *Sma*I and *Pst*I and cloning into the *Sma*I and *Pst*I sites of pBluescript. The cyclin B cDNA clone in the vector pGem4Z, pCyclin B (Pines and Hunter, 1990), was the gift of Tony Hunter (Salk Institute, La Jolla, CA).

Expression of GST fusion proteins. GSTE7, GSTΔDLLC, GST20KΔDLLC, and GSTΔDLLC31F were expressed and purified as described (Guan and Dixon, 1991). GST–p107, GST–cyclin E, and GST–27C2GE7 were expressed and purified as described (Koff *et al.,* 1992).

Antibodies. The monoclonal anti-cyclin E antibody HE12 used in Western blots was a gift of Emma Lees (Massachusetts General Hospital Cancer Center; Boston, MA). The anti-cyclin E monoclonal antibody HE111 used in kinase assays was also a gift of Emma Lees and later was purchased as an agarose conjugate (Santa Cruz Biotechnologies, Santa Cruz, CA) for use in preclearing synchronized cell extracts. The polyclonal anti-cyclin A antibody was a gift from Tony Hunter. Anti-p107 monoclonal antibody SD-9 was a gift of Nicholas Dyson (Massachusetts General Hospital Cancer Center). The polyclonal antibody M-2, used to visualize cdk2 in Western blots was purchased from Santa Cruz Biotechnologies. Purified polyclonal anti-E7 antibody affE7 (McIntyre *et al.,* 1993) was used for *in vitro* binding studies. Polyclonal antibody 247 was raised against the peptide CEARIELVVESSA, corresponding to amino acids 68 to 82 of HPV-18 E7, which was linked to keyhole limpet hemocyanin (KLH) by standard techniques (Harlow and Lane, 1988). E7-specific antibody was affinity purified against a GST–E7 column as described in (Koff *et al.,* 1992) and then cross-linked to protein A–Sepharose with dimethylpimelidate (Harlow and Lane, 1988).

Cell culture. The HaCat and C33a cell lines were maintained in DMEM (JRH Biosciences, Lenexa, KS) with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY). Primary human foreskin keratinocytes were grown in KGM (Clonetics, San Diego, CA).

Identification of cellular proteins bound to GST–E7 via immunoblot. Asynchronously dividing cultures of primary human foreskin keratinocytes, C33a, or HaCat cells were harvested as above. Five milligrams of thawed extract was precleared with 8 μg GST and 100 μl of glutathione–agarose and then mixed with 5 μg GST–E7 or GST. Protein complexes were precipitated with 25 μl of glutathione–agarose, separated by SDS–polyacrylamide gel, and transferred to a PVDF filter. The filter was then probed with antibodies to cyclin E, cyclin A, p107, or Rb. Horseradish peroxidase-linked anti-rabbit or anti-mouse antibody was used as the secondary antibody, and antigen–antibody complexes were visualized via the ECL (Amersham, Arlington Heights, IL) chemiluminescent detection system.

To determine the cell cycle specificity of the interaction of cellular proteins with GST–E7, 5 mg of extract from C33a cells that had been synchronized and harvested as detailed above was mixed with 5 μg GST–E7 and processed as described below. The filter was cut into strips to separate proteins of different molecular weights and the appropriate strip was probed with antibodies to cyclin E, p107, or cdk2. The intensity of the chemiluminescent signal at each time point was determined by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

In vivo association. C33a cells were infected with the E7 expressing retrovirus pMV718E7 or the parental virus pMV7 (Gifts of Nissim Hay) and selected with 1 mg/ml

G418 for 1 week to generate the cell lines C3318E7 and C33pMV7. Extracts from these cell lines, C33a, or HeLa cells were prepared as described and mixed with protein A–Sepharose-conjugated anti-cyclin E or anti-E7 antibody. Bound proteins were resolved by SDS–PAGE, transferred to Immobilon-P membranes, and analyzed by immunoblotting.

In vitro mixing of E7 with GST–p107 and GST–cyclin E. Five micrograms of bacterial-expressed, nonfused E7 (McIntyre *et al.,* 1993) was mixed with 0.25, 0.5, 1, or 5 μg GST–p107 or GST–cyclin E in a total of 200 μl ELB. After mixing for 1.5 hr at 4°, complexes were collected with 25 μl glutathione–agarose. Bound protein was eluted in sample buffer, electrophoresed on a 12.5% SDS–polyacrylamide gel, and transferred to nitrocellulose. The blot was probed with affE7 using an alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody. Complexes were visualized colorimetrically with an alkaline phosphatase conjugate substrate development kit (Bio-Rad, Hercules, CA).

Coprecipitation of in vitro translated cyclin E with GST–E7. Cyclins E and B were transcribed and translated *in vitro* with the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) to produce [^{35}S]methionine-labeled proteins. Twenty-five microliters of each translation reaction was mixed with 1 μg of GST–E7 and 5 μg GST–p107 or GST in a total volume of 300 μl ELB. Mixtures were precleared with 100 μl protein A–Sepharose in ELB and then the complexes were precipitated with affE7 and 25 μl protein A beads. Bound proteins were separated by SDS–PAGE and labeled cyclins E and B coprecipitating with GST–E7 were visualized on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Synchronization of C33a cells. C33a cells were synchronized at the G1/S boundary by treating with 2 m*M* mimosine (Sigma, St. Louis, MO) for 18 hr. Upon removal of the drug, cells were either harvested or refed with medium without mimosine. Cells were harvested every 2 hr for a total of 16 hr subsequent to the removal of the drug and lysed in 1 ml ELB lysis buffer (72) (50 m*M* HEPES, pH 7.0, 250 m*M* NaCl, 5 m*M* EDTA, 10 μ*M* $ZnCl_2$, 100 m*M* NaF, 200 μ*M* Na_3-*o*-VO_4, 1 m*M* DTT, 1 m*M* PMSF, 1 m*M* benzamidine HCl, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 0.5% NP-40) per plate. Clarified lysates were stored at −80°. Duplicate plates of cells were synchronized in the same manner but fixed in 75% ethanol for cell cycle analysis. Prior to analysis, fixed cells were pelleted and resuspended in propidium iodide staining buffer (3.8 m*M* sodium citrate, 0.125 mg/ml RNase A, 10 mg/ml propidium iodide). After 1 hr, the DNA content of the cells was determined by flow cytometry using a fluorescence-activated cell sorter (FACS) and CellFit software.

Kinase assays. To determine the kinase activity associated with wild-type and mutant GST–E7 fusion proteins, 1-mg extracts of asynchronously growing C33a cells were prepared and mixed with 1 μg wild-type or mutant GST–E7 as described for the immunoblots. Cyclin E was also precipitated from extracts with the anti-cyclin E antibody HE111. Beads were washed twice with ELB and then twice more with kinase buffer (50 m*M* HEPES, pH 7.0, 15 m*M* $MgCl_2$, 1 m*M* DTT) plus 0.1 mg/ml BSA. Kinase reactions were then performed in 50 μl kinase buffer with 10 μCi [γ-^{32}P]ATP and 25 μ*M* cold ATP at 30° for 30 min. Reactions were stopped by the addition of an equal volume of 4× Laemmli sample buffer and separated on a 7.5% SDS–PAGE gel. Labeled proteins were visualized by autoradiography.

Histone H1 kinase activity following cyclin antibody preclearing. Five hundred micrograms of cell extract was precleared twice with cyclin A antibody BF683 (Santa Cruz), cyclin E antibody HE111 (Santa Cruz), or purified mouse IgG1 isotype control 03001D (PharMingen) and immune complexes were collected with protein G–Sepharose (Pharmacia) before determining the kinase activity associated with GSTE7, GST, cyclin A, and cyclin E using histone H1 as a substrate by previously described methods (Koff *et al.,* 1991). Following SDS–PAGE and autoradiography, radioactivity incorporated into histone H1 was quantitated by a phosphorimager (Fuji, Japan).

RESULTS

Cyclins A and E bind GST–E7. To investigate if cyclin E can associate with HPV 18 E7, we examined the ability of proteins from cell extracts to bind to an N-terminal fusion of E7 with glutathione *S*-transferase (GST–E7). For these studies, equal amounts of purified bacterially expressed GST–E7 fusion protein were mixed with unlabeled extracts from a variety of HPV-negative human epithelial cells including normal human foreskin keratinocytes (HFK), HaCat and C33a cells. Proteins bound to GST–E7 were then examined by Western blot analysis using antibodies to cyclin E. Three forms of cyclin E with molecular weights of 45 to 55 kDa were observed in whole cell extracts (WCE) of HFK or C33a cells (Fig. 1A), consistent with previous observations (Koff *et al.,* 1991; Dulic *et al.,* 1992). In contrast, in the HaCat cell line, only a single predominant form of cyclin E was observed (Fig. 1A). GST–E7 was found to preferentially bind the slowest migrating form of cyclin E, while only a low level of association was observed with the other forms (Fig. 1A). This preferential association was observed in extracts from all three cell lines. The ability of GST–E7 to bind cyclin A was also examined by a similar protocol and the results are shown in Fig. 1B. The association of GST–E7 with cyclin A was observed in extracts from all three cell lines consistent with previous observations (Tommassino *et al.,* 1993). Similarly, the association of E7 with p107 was also observed as previously reported (Davies *et al.,* 1993; Ciccolini *et al.,* 1994) with the highest level of binding detected in C33a cells (not shown).

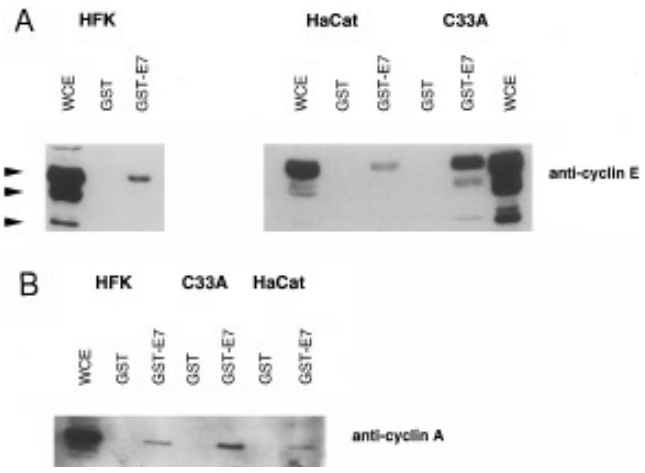


FIG. 1. Western blot analysis of cyclin E and cyclin A bound by GST–E7 in a variety of cell lines. (A) Multiple forms of cyclin E present in whole cell extracts (WCE) from human foreskin keratinocytes (HFK), HaCat, and C33a are shown. Proteins precipitated by GST–E7 or GST are indicated. Following separation by SDS–polyacrylamide gel electrophoresis, proteins were transferred to a membrane and probed with antibodies to cyclin E. (B) Similar analyses as in A but with antibodies to cyclin A. Proteins were visualized via chemiluminescence using horseradish peroxidase-conjugated secondary antibodies.

Cyclin E binds E7 in vivo. While GST fusion methodologies provided a simple way of studying E7-associated proteins, it was important to further confirm that the binding of cyclin E to E7 occurred *in vivo.* Therefore, we performed immunoprecipitation analyses using HeLa cells, which express HPV 18 E6 and E7, as well as pMV7–18E7 cells, which were derived following infection of C33a cells with E7-expressing amphotropic retroviruses. Cell lysates were incubated with affinity-purified antibodies directed against the C-terminus of E7 (247) or antibodies directed against cyclin E (HE111). Following immunoprecipitation, the bound proteins were examined by Western analysis. All three forms of cyclin E were immunoprecipitated with cyclin E antibodies (HE111) from HeLa and C33a cells (Fig. 2A). Using antibodies to E7, a specific association was detected between the endogenous E7 and the slowest migrating form of cyclin E in HeLa cells, consistent with our observations using GST–E7. No cyclin E proteins were immunoprecipitated with the E7 antibody from C33a cells which do not express E7 (Fig. 2A). A similar association of the slowest migrating form of cyclin E with E7 was also observed in immunoprecipitations of pMV7–18E7 cells but was not found in cells which had been infected with a control retrovirus (pMV7; Fig. 2B). We next examined if the kinase, cdk2, which is usually found in association with cyclin E, was also present in complexes with E7. The Western blot shown in Fig. 2B was reprobed with antibodies to cdk2, and the presence of cdk2 in association with E7 was observed (Fig. 2B). It is likely that the binding of cdk2 occurs through the association of E7 with both cyclins E and A. We were unable to coprecipitate significant levels of E7 with antibodies to cyclin E (Fig. 2B) which may be the result of low levels of interaction or blocked epitopes. The experiments described above demonstrated that we could precipitate a complex of E7 and cyclin E from cell lines, which confirmed our previous observations using GST fusion proteins. Since the use of immunoprecipitations to study E7/cyclin E interactions was not efficient, we chose to use GST–E7 proteins for the subsequent studies.

E7 associates with cyclin E through p107. We next examined the ability of E7 and cyclin E to interact directly *in vitro* using purified, nonfused E7 and GST–cyclin E. As a positive control, we first examined the binding of GST–p107 to purified E7. The amount of E7 bound by GST–p107 was found to correlate with input levels (Fig. 3A), but no E7 protein was bound by either GST–cyclin

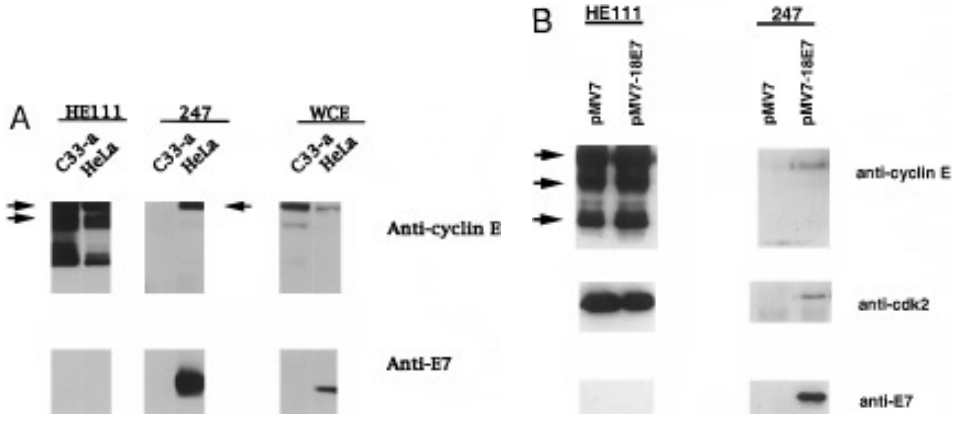


FIG. 2. Cyclin E and HPV 18 E7 associate *in vivo.* (A) HPV 18 E7 or cyclin E proteins were immunoprecipitated from HeLa cells with anti-E7 antibody 247 or anti-cyclin E antibody HE111. Proteins were separated by SDS–PAGE, transferred to a membrane, and identified by probing with anti-cyclin E or anti-E7 antibodies followed by visualization with chemiluminescence. Multiple forms of cyclin E are indicated and E7 associates with the slowest migrating form. (B) Extracts from C33a cells infected with the HPV 18 E7 expressing retrovirus pMV7–18E7 or parent virus pMV7 were immunoprecipitated with antibodies to E7 or cyclin E as described under Materials and Methods. Associated proteins were identified by Western analysis using anti-cyclin E, anti-E7, or anti-cdk2 antibodies.

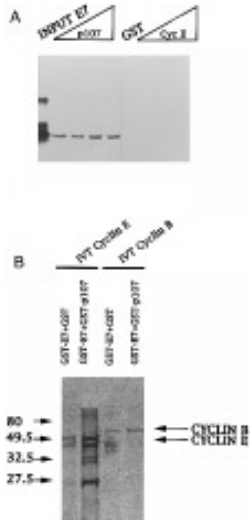


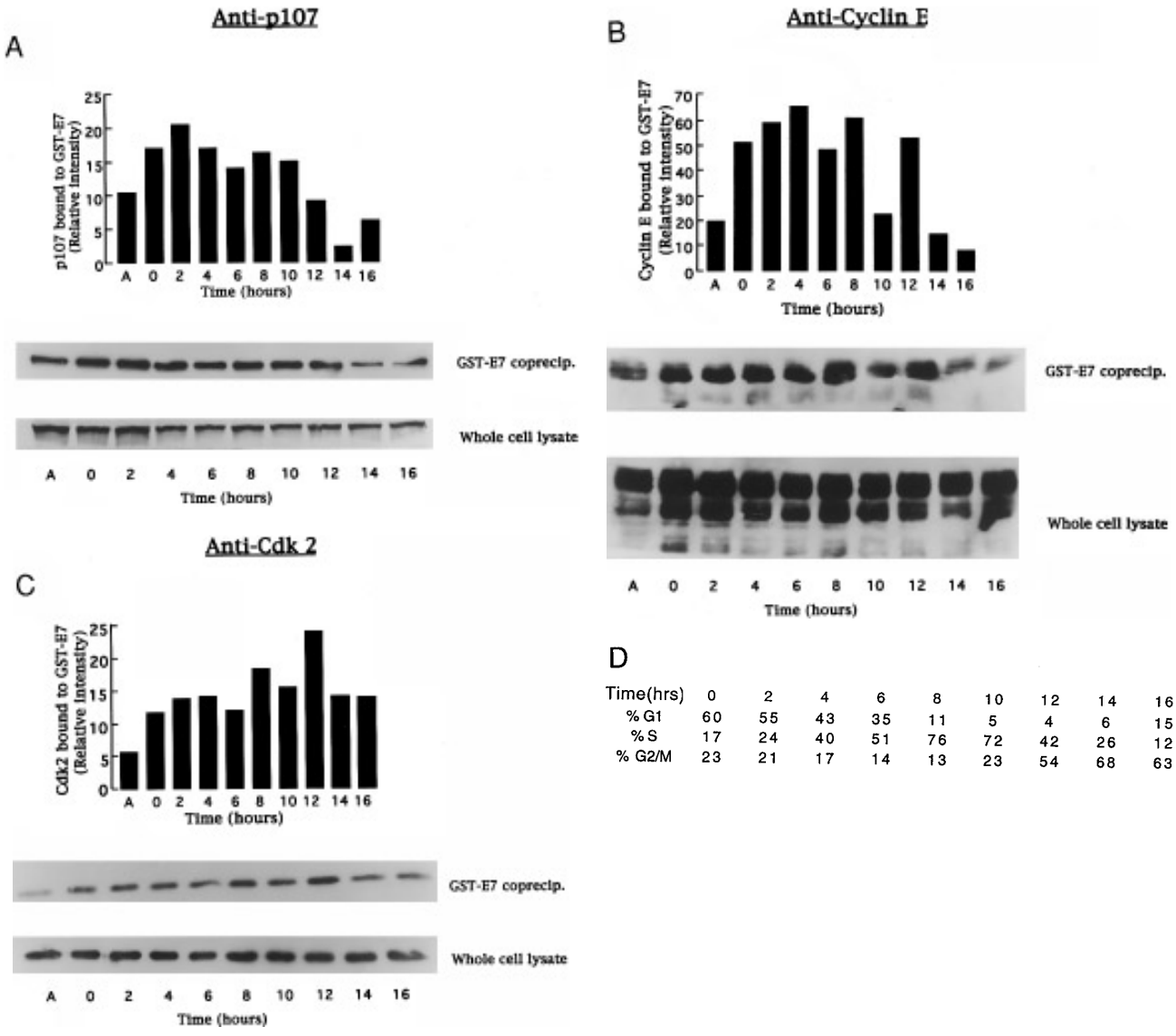
FIG. 3. *In vitro* association between purified E7 and p107 or cyclin E. (A) Bacterial expressed and purified E7 was mixed with increasing amounts of GST–p107 or GST–cyclin E. E7 protein was also mixed with GST as a control. Complexes were precipitated with glutathione agarose and bound E7 was detected by Western analysis. Lane 1 shows input E7. (B) Cyclins E and B were translated *in vitro* and labeled with [^{35}S]methionine. Labeled proteins were mixed with GST–E7 and GST–p107 or GST. Complexes were immunoprecipitated with affE7 and separated by SDS–PAGE. Labeled protein bound to E7 was visualized with a Molecular Dynamics phosphorimager.

E or the GST control. This suggested that E7 and cyclin E do not bind directly. By SDS–polyacrylamide gel analysis, the majority of GST–cyclin E in our preparations was full length excluding the possibility that proteolysis of the E7 binding domain could be responsible for the failure of the two proteins to form a complex (data not shown).

Our studies examining the binding of cyclin E to GST–E7 in different cell extracts demonstrated the highest level of association in C33a cell extracts (not shown), which also contained significant amounts of p107 (Lees *et al.,* 1992). This indicated that p107 might play a role in cyclin E/E7 complex formation similar to what has been suggested previously for cyclin A binding to E1A (Faha *et al.,* 1992). To determine if p107 mediates the association of E7 with cyclin E, *in vitro* translated cyclin E was mixed with GST–E7 in the presence or absence of GST–p107 and complexes were immunoprecipitated with E7 polyclonal antibodies. Consistent with the above observations, GST–E7 did not bind *in vitro* translated cyclin E above background levels observed with GST alone (Fig. 3B). In contrast, the addition of GST–p107 was found to dramatically increase the amount of cyclin E that was coprecipitated with E7 (Fig. 3B, compare lanes 1 and 2). The faster migrating species bound by p107 seen in lane 2 are truncated cyclin E products which are most likely the result of internal initiation of translation. To control for nonspecific binding, cyclin B, which does not bind E7, was translated *in vitro* and examined for its ability to bind GST–E7. As seen in Fig. 3B, a low level of nonspecific binding was observed that was similar in amount to that seen with GST alone. No increase in cyclin B binding was observed upon the further addition of GST–p107. The E7 antibodies also failed to precipitate GST–p107/cyclin E complexes in the absence of added GST–E7 (not shown). We conclude that cyclin E binds E7 indirectly through p107.

Cell cycle dependence of E7 association with p107 and cyclin E. Since the levels of cyclin E vary throughout the cell cycle, we next investigated the distribution of E7 binding activities during the cell cycle. For these studies, C33a cells were synchronized in late G1 with the drug mimosine and then released by the addition of medium lacking the drug. Cellular extracts were prepared every 2 hr following release for a total of 16 hr, and FACS analysis of control cells confirmed that the majority of the cells were in G1 after treatment with the drug (Fig. 4). Following release from the block, the cells progressed synchronously into S phase at 8 hr, and after 14 hr the majority of the cells were in G2/M. Cell extracts were prepared at the various time points and mixed with GST–E7, complexes were isolated, and the levels of bound p107 and cyclin E were examined by Western blot analysis. p107 was found to bind GST–E7 at a relatively constant level through the early part of the cell cycle but diminished when cells entered G2/M (Fig. 4A). This correlated with a general decrease in the total level of p107 in the cells in G2/M and further demonstrated that association of E7/p107 is important for cyclin E binding. Similar to our previous observations, E7 was found to bind only the slowest migrating form of cyclin E which was highest from G1 through S and this binding was significantly reduced in G2/M (Fig. 4B). Cdk2 was observed to bind to E7 at a constant level at all time points examined (Fig. 4C) and reflects contributions of both E7/cyclin A and E7/cyclin E complexes.

E7/cyclin E complexes phosphorylate p107. The HPV 16 E7 protein has previously been reported to exhibit a kinase activity that is dependent upon sequences within the CR2 domain (Davies *et al.,* 1993). Based on our findings this activity could be due either to the binding of cyclin A/cdk2, cyclin E/cdk2, or both. Since our analyses indicated that E7 bound cyclin E through p107, it seemed possible that the loss of kinase activity associated with mutations in CR2 could be due to an inability to bind p107 and consequently cyclin E. To test this hypothesis, HPV 18 E7 CR2 mutants were constructed which were similar to the previously described HPV 16 E7 mutants (Davies *et al.,* 1993). These mutants were examined for their ability to bind GST–p107 using *in vitro* translation assays. Similar to results obtained with HPV 16, mutants of HPV 18 E7 in the CR1 domain and the C-terminal zinc finger region retained p107 binding activity (Figs. 5A and 5B). In contrast, mutants containing deletions in the Rb binding domain (ΔDLLC) as well as changes at amino acid 27 (27C2G) were unable to bind p107 efficiently



D

Time(hrs)	0	2	4	6	8	10	12	14	16
% G1	60	55	43	35	11	5	4	6	15
% S	17	24	40	51	76	72	42	26	12
% G2/M	23	21	17	14	13	23	54	68	63

FIG. 4. Analysis of changes in the levels of p107 (A), cyclin E (B), and cdk2 (C) throughout the cell cycle as well as changes in their association with GST–E7. Extracts of C33a cells that had been blocked at the G1/S boundary with mimosine and then released from the block for the indicated periods of time were incubated with the various GST–E7 proteins. "A" designates asynchronously dividing cells. Whole cell lysates from the different time points were analyzed by Western analysis with the corresponding antibody to p107, cdk2, or cyclin E. Proteins associated with GST–E7 were precipitated and analyzed in a similar manner. Proteins were visualized by chemiluminescence. The changes in the intensity of the signal at the different time points were determined by laser scanning densitometry and are presented in bar graph form. (D) Cell cycle distribution of cells as determined by FACS analysis.

(Figs. 5A and 5B). The corresponding GST fusions of these E7 mutants were then constructed and used to examine the kinase activities of E7-associated proteins. Equivalent amounts of purified GST–E7 proteins were mixed with extracts from C33a cells, and precipitated E7 complexes were examined for kinase activity following the addition of labeled γ-ATP. The pattern of phosphorylation of proteins complexed with wild-type and mutant GST–E7 proteins is shown in Fig. 5C. Among the phosphorylated proteins bound to wild-type E7 are proteins of molecular weight consistent with p107, p130, and an unidentified protein of molecular weight which we estimate to be greater than 150 kDa. These proteins were also phosphorylated in immunoprecipitated cyclin E complexes (Fig. 5C), which phosphorylated additional unidentified cellular proteins.

The GST–E7 mutants lost some or all associated kinase activity depending on the particular amino acids that were modified (Figs. 5B and 5C). The loss of p107 phosphorylation was observed in all CR2 mutants examined and correlated with the inability to bind p107 *in vitro* (Figs. 5B and 5C). Similar correlations of kinase activity with binding of p107 were observed by Davies *et al.* (1993) using histone H1 as a substrate. Phosphorylation

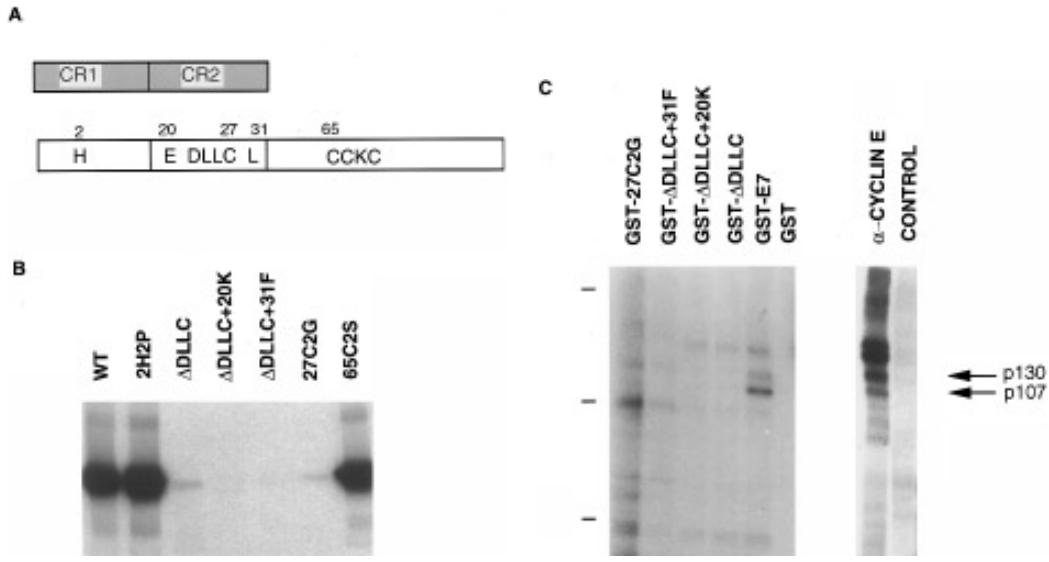


FIG. 5. Kinase activity associated with wild-type and mutant E7 proteins when mixed with extracts of C33a cells. (A) Locations of mutations introduced into HPV 18 E7. (B) Ability of E7 mutants to bind GST–p107. RNAs encoding mutant E7 genes were translated *in vitro* and labeled proteins mixed with GST–p107. Precipitated proteins were examined by autoradiography following precipitation and SDS–PAGE. (C) The indicated wild-type and GST–mutant E7 fusion proteins were mixed with extracts of asynchronously dividing C33a cells, complexes were precipitated with glutathione agarose, and *in vitro* kinase assays were performed. Similar kinase assays were performed on immune complexes using a control antibody (C), or anti-cyclin E. Phosphorylated proteins were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. Molecular weight markers (80, 106, and 205 kDa) are shown to the left, and phosphorylated p107 and p130 are indicated by arrows.

of p130 was observed at a very low level with the GST–ΔDLLC + 31 mutant but was not seen with the deletion mutants GST–ΔDLLC and GST–ΔDLLC + 20K. The ability to bind and phosphorylate the novel 150-kDa protein was lost with the GST–27C2G and GST–ΔDLLC + 31F mutants. This indicates that the sequences required by HPV 18 E7 to bind p107 may be slightly different than those required to bind p130 and p150. Similar patterns of phosphorylation of E7-associated proteins were also seen with extracts of normal human keratinocytes, although the level of GST–E7-associated p107 phosphorylation was reduced due to the lower levels of p107 expression in these cells (data not shown). These studies demonstrate that p107 and p130 protein binding correlates with kinase activity of complexed proteins as well with histone H1 activity.

A significant portion of E7-associated kinase activity is dependent upon bound cyclin E. We next sought to determine the contribution of E7/cyclin E/cdk2 complexes to the total kinase activity associated with E7. For these studies, we used histone H1 as a substrate since it was easily quantifiable by phosphoimager analysis following SDS–PAGE and has been used extensively in previous studies (Koff *et al.,* 1992). The relative histone H1 kinase activities of cyclin E and A complexes from normal human keratinocytes were first compared following immunoprecipitation of cell extracts with the corresponding antibodies (Fig. 6). Consistent with the majority of cells being in G0/G1, cyclin E-associated kinase activity was found to be greater than cyclin A activity (Fig. 6). When extracts were precleared two times with cyclin E antibodies prior to assaying for histone H1 kinase activity, the cyclin E-associated kinase activity was reduced by approximately 85% (Fig. 6). The kinase activity associated with E7 was next examined following incubation of extracts with GST–E7 and precipitation with glutathione beads. The total kinase activity associated with E7 by this method was found to be approximately 20% the activity seen in cyclin E immunoprecipitates. When keratinocyte extracts were first precleared twice with cyclin E antibodies, E7-associated kinase activity was reduced by 43% (Fig. 6). However, when similar extracts were precleared with cyclin A antibodies, the E7-associated kinase activity decreased by 30%. The residual activity associated with E7 following preclearing with cyclin E antibodies could be the result of cyclin A/cdk2, uncleared cyclin E/cdk2, or other as yet unidentified kinases. We therefore conclude that a significant portion of the kinase activity that is associated with E7 is due to the binding of cyclin E/cdk2.

DISCUSSION

The binding of cellular factors by the E7 proteins from the high-risk HPV types is critical to the processes of transformation and viral pathogenesis. Among the proteins which E7 binds is the retinoblastoma protein, Rb, as well as p130, p107, and cyclin A. In this study, we have identified cyclin E as another E7-associated protein. E7 does not bind cyclin E directly, but appears to bind

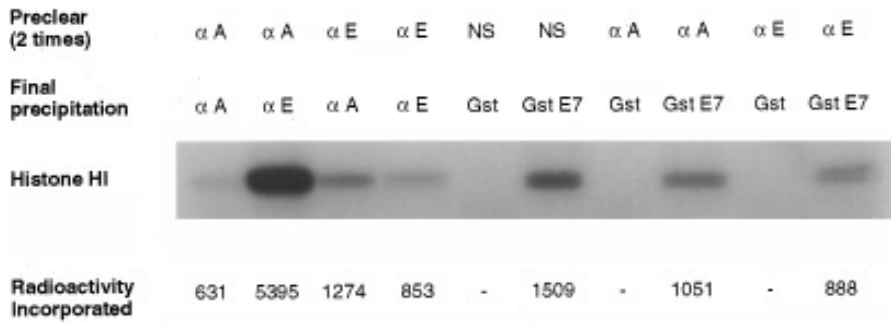


FIG. 6. Cyclin E kinase activity associated with E7. Whole cell extracts from normal human foreskin keratinocytes were precleared twice with either antibodies to cyclin A (αA), cyclin E (αE), or nonspecific antibodies (NS). The precleared extracts were then subjected to a final precipitation using either the cyclin antibodies, GST, or GST–E7. Precipitated complexes were incubated with histone H1 and labeled ATP and then the amount of H1 phosphorylated was examined following separation on an SDS–polyacrylamide gel. The amount of H1 phosphorylation was determined by phosphoimager (Fuji, Japan) analysis and is indicated below each lane.

indirectly through p107. This observation contrasts with the direct binding of cyclin A to E7 (Tommassino *et al.,* 1993). Several alternatively spliced forms of cyclin E are found in cells and E7 was found to associate with only the slowest migrating species. Since the function of the various forms of cyclin E has not yet been established, the significance of this specific association is not understood. Cdk2 was also found in association with E7 which is consistent with the observed E7-associated kinase activity. Among the proteins found to be phosphorylated by the cyclin E/E7/cdk2 complex were proteins of molecular weights consistent with p107 and p130 as well as an unidentified protein with a molecular weight of approximately 150 kDa. Our identification of these proteins is based primarily on similarities of molecular weight. The phosphorylation of p130 and p150 by the E7-associated kinase was observed with several E7 mutants which had lost the ability to bind p107 indicating that additional kinases are associated with E7 in the absence of p107 interaction. One possibility is that p130 and p150 also associate with cyclin E/cdk2 or cyclin A/cdk2 complexes and that they provide the residual kinase activity. Alternatively, it is possible that an unidentified kinase binds E7 and is responsible for p130 and p150 phosphorylation. The observation of a novel cellular protein, p150, which binds to sequences within the Rb binding domain of E7 suggests that it may represent a previously uncharacterized member of the Rb-related protein family.

The functional significance of E7's association with cyclin E most likely centers on the kinase activity of the complex. Preliminary data suggest that the cyclin E/cdk2/E7 complex exhibits kinase activity throughout the cell cycle with a slight peak at S while the E7/cyclin A/cdk2 kinase is most active in G2/M (McIntyre and Laimins, unpublished data). These observations are in agreement with studies involving E1A, which demonstrated a kinase activity during both S phase and G2/M due to contributions from both cyclin A–cdk2 complexes and cyclin E–cdk2 complexes (Faha *et al.,* 1993; Giordano *et al.,* 1991). Several possibilities exist for the function of E7/cyclin E/cdk2 complexes. For instance, these complexes could alter the normal time during the cell cycle at which the cyclin/kinase is active. Alternatively, the spectrum of proteins phosphorylated by the kinase complexes could be modified and in particular new proteins could be phosphorylated by the E7 complexes. Another function of E7 binding to cyclin E complexes could be to alter the action of cyclin/cdk2 regulatory proteins such as the recently identified p21 protein. Following exposure of cells to DNA damaging agents or radiation, p53 levels increase which results in the activation of p21 expression. Increases in p21 levels result in repression of cyclin/cdk2 kinase activity leading to cell cycle arrest (El-Diery *et al.,* 1993). E6-expressing cells contain reduced levels of p53 and fail to undergo cell cycle arrest following radiation treatment (Kessis *et al.,* 1993) due to an absence of p21. Interestingly, cells expressing E7 alone also fail to undergo cell cycle arrest following radiation treatment (Demers *et al.,* 1994; Slebos *et al.,* 1993) suggesting that E7 by itself may alter the cyclin response to kinase regulatory proteins.

One identified target of cyclin E/E7/cdk2 activity appears to be p107, and the resultant interactions may be important for transcriptional control. Lam *et al.* (1994) examined cells expressing E7 for the expression of b-myb, which is normally dependent on E2F-responsive sites bound by the S-phase-specific p107/cyclin A/E2F complex. In these cells b-myb expression was deregulated and no longer cell cycle dependent. The phosphorylation of p107 by E7 complexes could provide a mechanism for this activity. In contrast to the binding of E7 to Rb, the association of E7 with p107 does not result in dissociation of E2F. Instead, E2F remains complexed with p107/E7 (Arroyo *et al.,* 1993; Lam *et al.,* 1994) which suggests that E7 may alter the p107-mediated regulation of E2F by alternative mechanisms such as through changes in phosphorylation. Such alterations could contribute to transformation as well as to the normal activity

of E7 during a viral infection. In normal epithelia, cellular replication is limited to basal cells, and nuclei are degraded as cells differentiate. In an HPV infection, basal cells are induced to hyperproliferate and differentiated cells are thought to be arrested in G0 or G1 with nuclei intact. In the suprabasal region, infected cells are most likely driven into S phase to allow for genome amplification. Since entry into S phase is controlled by cyclin E, complex formation with E7 may also be important for this process. The targeting by E7 of proteins involved in cell cycle regulation is likely to be essential for the mechanism by which the high-risk HPV types contribute to malignancy as well as to the process of viral pathogenesis.

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